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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/53 | A1

(11) International Publication Number: WO 98/05962

(43) International Publication Date: 12 February 1998 (12.02.98)

(21) International Application Number: PCT/US97/13538

(22) International Filing Date: 1 August 1997 (01.08.97)

(30) Priority Data:
60/023,034 2 August 1996 (02.08.96) US

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(81) Designated States: European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR QUANTITTATING COMPETITIVE BINDING OF MOLECULES TO PROTEINS UTILIZING FLUORESCENCE POLARIZATION

(57) Abstract

The system comprises mixing a fluorescence-emitting compound that binds to the steroid hormone receptors in a solution containing the steroid hormone receptors. Then, measuring the fluorescence polarization of the solution. Subsequently, incubating the solution with at least one molecule that may compete with the compound for interaction with the steroid hormone receptors. Measuring the fluorescence polarization of the solution again. Finally, comparing the fluoresence polarization measurements to quantify any competitive interaction.

A Method For Quantitating Competitive Binding Of Molecules To Proteins Utilizing Fluorescence Polarization

Field

The field of the invention relates to the detection of molecules which bind to steroid hormone receptors by measuring the fluorescence polarization emission from a molecule. In particular, fluorescence-emitting compounds are used in competitive assays to measure binding of molecules to steroid hormone receptors such as estrogen receptor.

Background Of The Invention

The use of labeled oligonucleotides as probes in molecular analysis has been an important technique in molecular biology. Oligonucleotides have been labeled with radioisotopes, enzymes or fluorescent molecules. Because of the relatively low molecular weights of oligonucleotides, and the common availability of instrumentation for their automated synthesis, oligonucleotides are often used in blot-hybridization procedures or in gel-retardation assays for the detection and qualitative evaluation of macromolecules with which they may associate. These macromolecules may be either proteins, RNA molecules or DNA molecules.

Furthermore, an assay has been described in a publication that does not require a separation step. It is based on a modified fluorescent hormone (Hwang et. al. Biochemistry 31:11536-45, 1992). The assay uses the observation that when the hormone and receptor bind, the fluorescence intensity of the hormone decreases proportional to binding. Free hormone has a high fluorescence intensity and the bound hormone has a low fluorescence intensity. However, several components of the binding mixture can affect the intensity other than the receptor. The polarity of the solvent and non-specific binding molecules can have significant affects on the intensity, which can be incorrectly interpreted as hormone/receptor binding.

Another detection method described in the early 1900's utilizes fluorescence polarization. Fluorescence polarization assay techniques are based on the principle that a fluorescently labeled compound will emit fluorescence when excited by plane polarized light, having a degree of polarization inversely related to its rate of rotation. If the labeled molecule remains stationary throughout the excited state it will emit light in the same polarized plane; if it rotates while excited, the light emitted is in a different plane.

For example, when a large labeled molecule is excited by plane polarized light, the emitted light remains highly polarized because the fluorophore is constrained (by its size) from rotating between light absorption and fluorescent light emission. When a smaller molecule is excited by plane polarized light, its rotation is much faster than the large molecule and the emitted light is more depolarized. The emitted light has a degree of polarization that is inversely proportional to the molecular rotation. Therefore, small molecules have low polarization values and large molecules have high polarization values.

Fluorescence polarization assays are homogeneous in that they do not require a separation step such as centrifugation, filtration, chromatography, precipitation or electrophoresis. Assays can be performed in real time, directly in solution and do not require an immobilized phase. For example, fluorescence polarization has been used to measure enzymatic cleavage of large fluorescein labeled polymers by proteases,

Also, a kit is provided utilizing the methods listed above for identifying natural and non-natural molecules which bind to human steroid hormone receptors, for use in treating related diseases. The kit comprises instructions for utilizing fluorescence polarization to identify the molecules; a receptacle containing human steroid hormone receptors; and,

a receptacle containing fluorescence-emitting hormone which binds human steroid hormone receptors. The kit may further contain a receptacle containing a fluorescence-labeled nucleic acid

Reference is now made in detail to the preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings.

Brief Description of the Drawings

In the drawings:

FIG. 1 illustrates that the polarization of the fluorescent hormone is directly related to the concentration of estrogen receptor and the total shift is polarization is optimal with 535 nm emission.

FIG. 2 shows the results of fluorescent estrogen competition assay in single tube.

FIG. 3 charts the fluorescent estrogen competition assay in 96 well format.

FIG. 4 shows the specificity of fluormone binding to ER and BSA.

FIG. 5 plots the detection of ER binding to fluormone ES 1 and fluorescein labeled DNA in the same sample.

FIG. 6 depicts the detection of environmental compounds which bind estrogen receptor using a competitive fluorescence polarization assay.

FIG. 7 shows the stability of the ER/fluormone complex in 96 well plastic plates at room temperature.

FIG. 8 illustrates the determination of optimum fluorescent hormone for polarization assay and DMSO interference

FIG. 9 is a listing of compounds investigated with the method.

FIG. 10 is a chart listing small organic molecules and polarization values which can correct the fluormone ES1 polarization shift caused by DMSO.

cells of the body. The function of the different hormones is to control the activity levels of target tissues through their interaction with other molecules. A hormone, in general, is herein defined as any substance (natural or synthetic) that may act to exert a physiological response in tissues or bind to molecules normally bound by the natural hormone.

The term "nucleic acid", "polynucleotide", "oligonucleotide" are defined as multiple nucleotides attached in the form of a single or double stranded polynucleotide that can be natural; or derived synthetically, enzymatically, and by cloning methods. The use of the terms RNA and DNA may be used interchangeably in this description and include either double- or single-stranded nucleic acids. However, the preferred embodiments refer to any form of DNA.

A Fluormone is a shortened term for 'fluorescent hormone'. It is any molecule that is covered by the hormone definition and emits fluorescence. Characteristics of a fluormone suitable for use with this method include:

- Emission light must be isotropic.
- The quantum yield must be sufficiently high over the potential background.
- The fluorescent molecule must be biochemically inert.
- The fluorescent signal must be stable in an aqueous buffer for a period of time sufficient for the assay to be performed.
- The fluorescent hormone must be stable in a plastic 96 or 384 well plate.
- The fluormone must have a low polarization value when free and a high polarization when bound.
- The fluormone must have a binding affinity tight enough to allow for competition assays.
- It must be compatible with solvents used in high throughput screens.

Fluorescence Polarization is fully described and defined in U.S. Patent [Serial No. 08/353,079] issued to Burke et al. and incorporated herein by reference. Fluorescence anisotropy can also be used to measure the binding. Like polarization values, it is

of time to allow binding and reach equilibrium. Then the polarization value is quantified using an appropriate instrument.

The instrument must be a fluorescence polarimeter device. Standard fluorescence plate readers or spectrometers cannot read polarization values, and instruments retrofitted for polarization are generally less sensitive than dedicated fluorescence polarization instruments. In the preferred embodiments, a single tube Beacon 2000 FP instrument (PanVera Corporation, Madison, WI) was used to quantify polarization.

An advantage of fluorescence polarization over fluorescence intensity is the ability to remove unrelated factors from the measurement, such as other molecules which adversely affect intensity. Advantages over other technologies include: 1) FP measurements are performed in solution, allowing molecules to be studied at equilibrium in contrast to other techniques which require the attachment of one of the binding partners to a solid support; 2) FP measurements can be taken every six seconds, making kinetic reactions (associations, dissociations, or enzymatic degradations) easy to follow in real time; 3) FP gives a direct measure of a fluorescence-emitting compound (tracer) bound/free ratio, separation is not required which eliminates filter binding, precipitation, and centrifugation steps; 4) manipulation or alteration of the samples is not required, therefore artifactual loss of signal through handling does not occur; 5) FP measurements do not deleteriously affect the sample – samples can be analyzed, treated, and reanalyzed. 6) FP measurements can be performed at temperatures from 6°C to 65°C.

In a preferred embodiment, a described method is used to detect molecules that may compete with a Fluormone to a specific receptor domain or interact with another part of the receptor that may affect the Fluormone's receptor binding domain. In another preferred embodiment, a described method allows analysis of how DNA binding to a receptor domain is affected by molecules binding to other receptor domains. The preferred receptor is a steroid hormone receptor and more specifically the human estrogen receptor.

competitor necessary to release ES1 from ER. As the fraction of unbound ES1 increases, the polarization value drops.

Certain nonsteroidal compounds also bind to the ER by way of competition with a natural hormone, estradiol for ER binding domains. Some of these compounds mimic the estrogenic activity of estradiol and have been termed "environmental estrogens." It is a concern that common chemicals such as herbicides and pesticides may enter the body and stimulate estrogen response pathways.

In a preferred embodiment of a competitive assay, a standard curve is developed using a bound fluormone and various concentrations of estradiol. This allows one to determine the amount of estradiol in unknown samples, including tissue samples, foodstuffs, or chemical samples.

In another preferred embodiment, a kit is provided which contains the reagents necessary to perform a competition assay to assess affinity of test compounds for ES. Instructions are provided indicating that ER is added to a solution containing ES1 to form a Fluormone/ER complex with high fluorescence polarization. The complex is then to be added to estrogen competitors in microtiter wells or 6 mm glass tubes. The shift in polarization in the presence of test compounds is used to determine relative affinity of test compounds for ER.

Examples

Example 1

The polarization of the fluorescent hormone is directly related to the concentration of estrogen receptor and the total shift is polarization is optimal with 535 nm emission.

The optimal excitation and emission wavelengths can change for a give fluorophore depending on which solvent is used. Some choices of wavelengths may give the maximum absorption and emission spectra but they may not give the optimal polarization data. Several combinations of excitation and emission filters were tested to determine which gave the optimal results.

were serially diluted in ES1 Binding Buffer. Fluormone ES1 and ER were then added to each tube to a final concentration of 1 nM and 5 nM respectively. The three components were allowed to reach equilibrium at room temperature and polarization values were measured using the Beacon 2000 FP instrument The data were analyzed using a curve fitting program in Graphpad Prism. (see FIG. 2)

Example 3

Fluorescent Estrogen Competition Assay in 96 Well Format

The experiment was performed as in Example 2 except that the assay required 20 nM ER and 20 nM Fluormone ES1 for the assay and the dilutions were done in 96 well plastic plates. The polarization values were measured (see FIG. 3) using a FPM-2 Fluorescence Polarization Instrument from Jolley Consulting and Research. Test compounds were serially diluted in ES1 binding buffer and added to wells containing the ES1/ER complex. The plates were allowed to equilibrate at room temperature for at least one hour before the polarization values were measured. The total volume in each well was 200 µl.

Example 4

Specificity of Fluormone binding to ER and BSA.

Human recombinant estrogen receptor or bovine serum albumin (BSA) were serially diluted in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Fluormone ES1 was added to a final concentration of 1 nM. The mixtures were allowed to reach equilibrium for 1 hour at room temperature and the polarization values were measured using a Beacon Fluorescence Polarization Instrument (see FIG. 4). Excitation was at 360 nm and emission was measured at 485 nm. The starting concentration of the ER and BSA were 200 nM and 60 uM respectively. The concentration of protein is measured in nM and the polarization is measured in mP. This data is significant in that there is over a 500 fold difference in the binding affinities and that even though the binding to BSA is weak, it is very specific and the top of the curve is flat.

The mixtures were allowed to equilibrate for 2.5 hours at room temperature and the polarization values were read 360/485 nm excitation/emission. The pesticides effectively competed the fluormone ES1 off the estrogen receptor reducing the polarization value (see FIG. 6) in going form the bound to free state.

Example 7

Stability of the ER/Fluormone Complex in 96 Well Plastic Plates at Room Temperature

Typically in a high throughput screen, 100,000 to 1,000,000 compounds are screened for their ability to bind the target molecule. Typically the target is an enzyme or a receptor-like binding partner. The 96 well plates are usually handled in groups of 50 and it is necessary that the assay is stable in the plates for period of time that it takes to 1) fill 50 plates, 2) allow the reaction to go to completion), 3) read the values in all the wells in the 50 plates. We needed an assay that was stable for up to 6 hours at room temperature. To achieve this stability, we tested a large number of compounds for their ability to improve the assay. The estradiol is serially diluted into 11 wells with five replicate rows. As described, the fluormone ES1/estrogen receptor mixture is added to each well and the binding reaction is allowed to reach equilibrium during one hour. The polarization values of the sample in each well is then measured (see FIG. 7). As the amount of estradiol is increased, it completes with the fluormone binding to the ER producing free fluormone which has a low polarization value. At each concentration of estradiol, data was taken at 1, 2, 4, 6, and 8 hours. The assay gives stable data over this time range at room temperature.

Example 8

Determination Of Optimum Fluorescent Hormone For Polarization Assay And DMSO Interference

Three fluorescent estrogens were tested in these experiments, THC-ester, THC-ketone, and THC-amide. Fluormone ES1 is the same as the THC-ester and is the fluorescent hormone in the kit. We examined all of these in several different ways: 1) which gave the highest intensity signal at a given concentration, 2) which gave the

complex, and 4) does it stabilize the reaction mixture in 96 well plastic plates at room temperature? Many of the compounds were

This list includes several classes of compounds include ionic salts, chaeotropic agents, ionic detergents, non-ionic detergents, organic solvents, proteins, buffers, sugars, and other carbohydrates. Many of these reagents were tested both individually and in combination with other compounds to determine if a class of compounds could be used to improve the assay. Several of the small organic compounds had a corrective effect on the DMSO problem interfering with the fluormone polarization. These compounds were tested further in Figure 10.

Example 10

Small Organic Molecules Can Correct the Fluormone ES1 Polarization Shift caused by DMSO

Several compounds were tested to determine if they could reverse the high polarization shift caused by DMSO. To test the compounds, the following reagents were added to a borosilicate test tube (6 mm OD) at room temperature:

100 µl of 10 mM Tris-HCl, pH 7.5

2 µl 400 nM THC-ester in ethanol

2 µl DMSO

10 µl of one of the compounds in Figure 10

The polarization values were then read using a Beacon 2000 Fluorescence Polarization Instrument. The dimethylformamide gave the best correction of the polarization value and was selected for use in the assay. It is not known why DMSO causes the upward shift in polarization nor why DMF reverses the polarization back down to its normal level. DMF was also tested and shown at concentrations necessary to block the DMSO effects, it did not significantly change fluormone ES1/ER binding.

Example 11

2.	40 nM Fluormone ES1	competitor⇒⇒⇒⇒	2.	20 nM Fluormone ES1	Polarization
3.	10 mM DTT		3.	5 mM DTT	Value
			4.	Competitor	

A competition curve will be generated by adding an aliquot of the 2X Fluormone ES1/ER complex to a serial dilution series of the test compound. The polarization will be plotted against test compound concentration. The concentration of the compound that results in a half maximum shift equals the IC_{50} (concentration of competitor needed to displace 50% of Fluormone) of the test compound.

Using the table below, make enough of the 2X complex for all of the reactions required. A volume of 50µL of 2X complex is needed for each reaction tube. The concentration of this 2X solution is 40 nM Fluormone ES1, 38 nM ER, and 10 mM DTT in Estrogen Screening Buffer. The Fluormone ES1 is dissolved in ethanol and is therefore quite volatile. Keeping this reagent on ice will not only aid in its stability, but reduce its volatility. 0.5 µL of the Fluormone ES1 and DTT is needed for each reaction. The ER concentration is variable, therefore the amount to add to make the 2X complex must be calculated using the following formula. Write in the number of reactions needed [A], the concentration of the ER (taken from the Certificate of Analysis) [B] and calculate the volume of ER in µL needed [C].

# of reactions as needed [A] _____ x 1.9 pmoles/reaction ÷ [ER] in pmole/µL [B] _____= µL of ER needed [C] _____.

The table below is a recipe for making the complex assuming the ER concentration is 1.2 pmole/µL.

Number of tubes [A]	4µM Fluormone ES1	ER [C]	1M DTT	Screening Buffer	Final Volume (50µL/reaction)
50	25µL	79µL	25µL	2.37ml	2.5ml
250	125µL	396µL	125µL	11.85ml	12.5ml

We Claim:

1 A method for measuring competitive binding activity of molecules to steroid hormone receptors, comprising:

a) mixing a fluorescence-emitting compound that binds to the steroid hormone receptors in a solution containing the steroid hormone receptors,

b) measuring the fluorescence polarization of the solution from step a),

c) incubating the solution of step a) with at least one molecule that may compete with the compound for interaction with the steroid hormone receptors;

d) measuring the fluorescence polarization of the solution during step c); and,

e) comparing the fluorescence polarization measurements of step b) with step d) to quantify any competitive interaction.

2. The method of claim 1 wherein the steroid hormone receptors are purified.

3. The method of claim 2 wherein the purified steroid hormone receptors comprises recombinant steroid hormone receptors.

4. The method of claim 1 wherein the quantitation comparison of step e) is of sufficient magnitude to be suitable for use with a screening assay.

5. The method of claim 4 wherein the screening assay is performed on a multi-well plate.

6. The method of claim 1 wherein the fluorescence-emitting compound comprises a hormone that inherently emits fluorescence.

7. The method of claim 1 wherein the steroid hormone receptors comprises estrogen receptor.

14 The method of claim 13 wherein the steroid hormone receptors comprises estrogen receptor.

15 The method of claim 8 wherein the nucleic acid comprises a deoxyribonucleic acid.

16 A kit utilizing the method of claim 1 for identifying natural and non-natural molecules which bind to human steroid hormone receptors, for use in treating related diseases, comprising:

a) instructions for utilizing fluorescence polarization to identify the molecules;

b) a receptacle containing human steroid hormone receptors; and,

c) a receptacle containing fluorescence-emitting hormone which binds human steroid hormone receptors.

17 The kit of claim 16 wherein the human steroid hormone receptors are purified

18 The kit of claim 17 wherein the human steroid hormone receptors are recombinant.

19. The kit of claim 18 further comprising a receptacle containing a fluorescence-labeled nucleic acid.

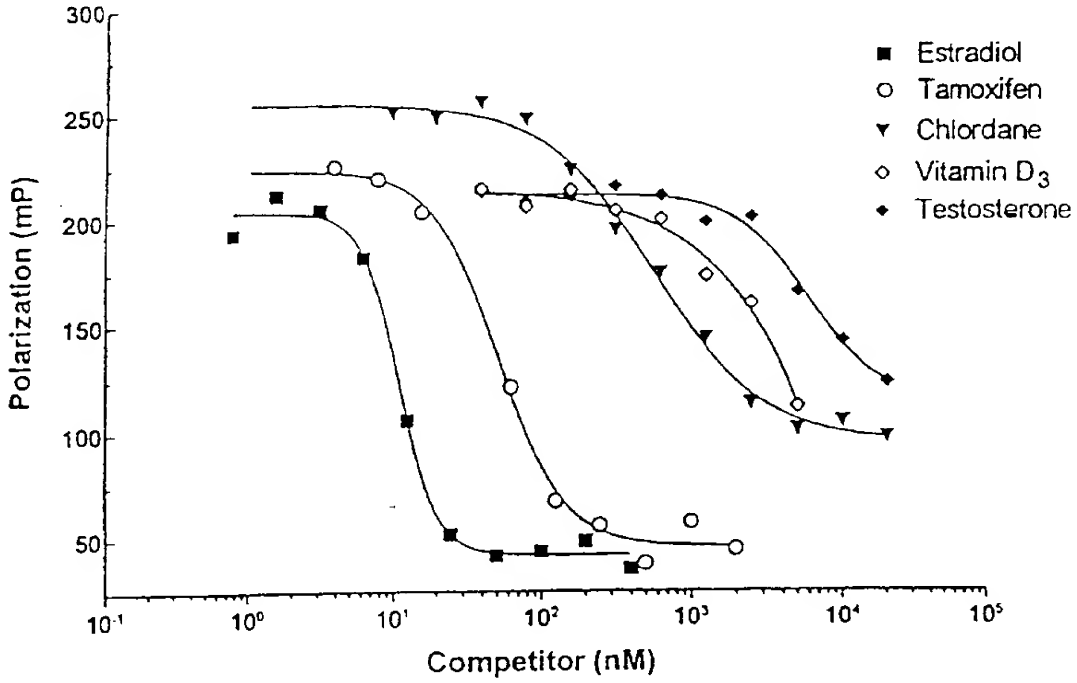
300
250
200
150
100
50
Polarization (mP)
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
Competitor (nM)
Estradiol
Tamoxifen
Chlordane
Vitamin D_3
Testosterone

Figure 2

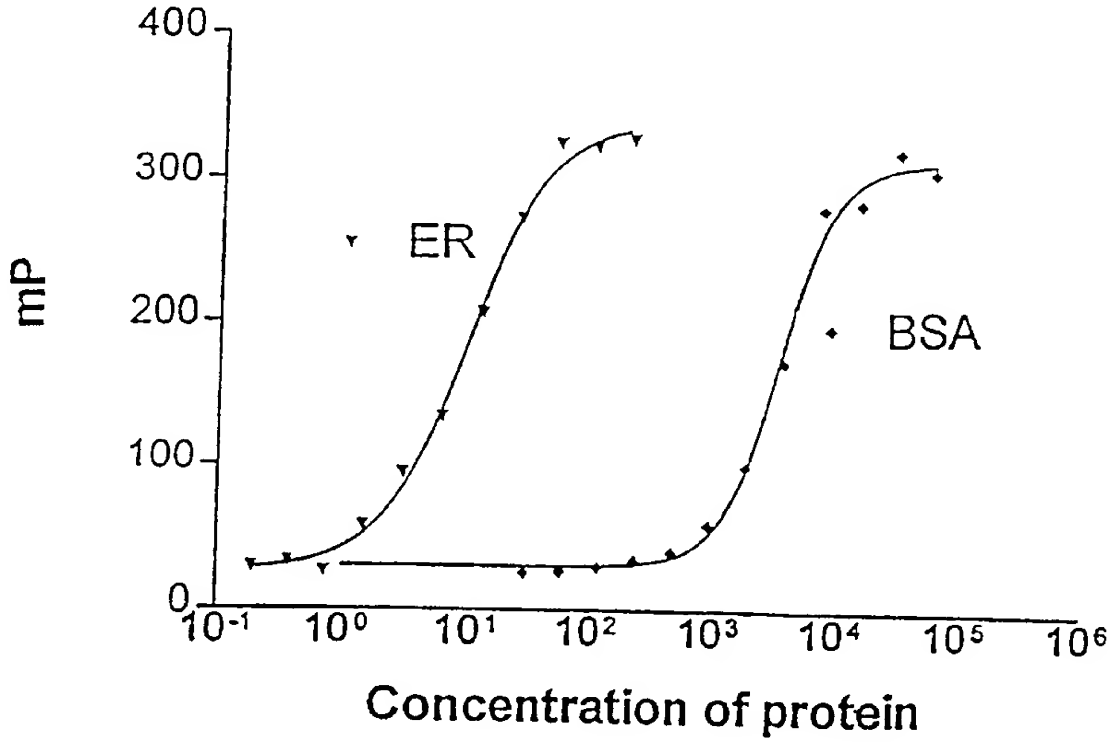
400
300
200
100
0
mP
ER
BSA
10-1
100
101
102
103
104
105
106
Concentration of protein

Figure 4

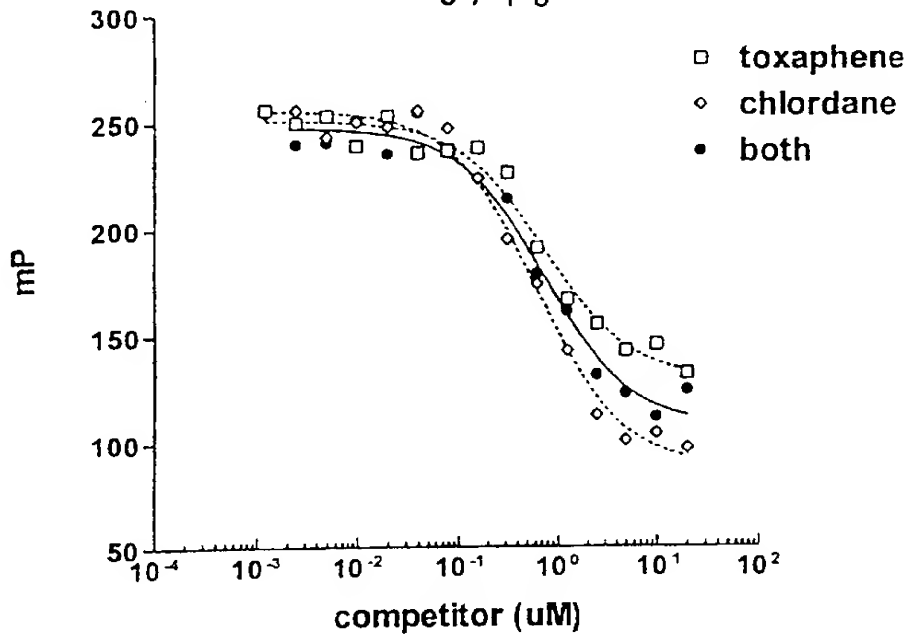
300
250
200
150
100
50
mP
toxaphene
chlordane
both
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
competitor (uM)

Figure 6

THC Ester Concentration (nM)	DMSO Concentration (%)	Polarization (mP)
20	25	232
20	12.5	251
20	6.25	226
20	3.13	164
20	1.56	148
20	0.78	99
20	0.39	81
20	0.20	52.9
20	0.10	62.5
20	0.05	40.7
20	0	48

THC Ketone Concentration (nM)	DMSO Concentration (%)	Polarization (mP)
15	5	206
15	0	137

THC Amide Concentration (nM)	DMSO Concentration (%)	Polarization (mP)
25	5	127
12.5	5	49.9
6.25	5	55.8
3.13	5	55

Figure 8

Average Starting Polarization (mP) of Fluormone ES1	79

Average Starting Polarization (mP) of Fluormone after Addition of 2% DMSO	253

Compound Added to Correct DMSO Effect on Fluormone ES1	Polarization (mP) after addition of Compound
N,N dimethylformamide	79
2- mercaptoethanol	120
acetonitrile	129
acetone	131
triethylamine	153
formamide	159
2 M KCl	159
ethylene glycol	161
butanol	162
0.2 M urea	181
1% sodium dodecyl sulfate	216
10 M formaldehyde	235
100 mM nickel chloride	238
1 M dithiothreitol	261
100 mM glycine, pH 2.0	276
1 M magnesium chloride	287
1 M sodium phosphate	340

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13538

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AUCOUTURIER, P. et al. Fluorescence Polarization Immunoassay of Estradiol. Diag. Immunol. 1983. Vol 1. pages 310-314, see entire reference.	1-19
Y	CHECOVICH, W. J. et al. Fluorescence Polarization- A New Tool for Cell and Molecular Biology. Nature. 18 May 1995. Vol. 375. pages 254-256, see entire reference.	1-19
Y	MIKSICEK, R. J. et al. Studies Using Fluorescent Tetrahydrochrysene Estrogens for in Situ Visualization of the Estrogen Receptor in Living Cells. Molecular Endocrinology. 1995. Vol. 9. pages 592-604, see entire reference.	1-19